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Development of a broadly protective modified-live virus vaccine candidate against porcine reproductive and respiratory syndrome virus

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ABSTRACT

Modified-live virus (MLV) vaccines are widely used to protect pigs against porcine reproductive and respiratory syndrome virus (PRRSV). However, current MLV vaccines do not confer adequate levels of heterologous protection, presumably due to the substantial genetic diversity of PRRSV isolates circulating in the field. To overcome this genetic variation challenge, we recently generated a synthetic PRRSV strain containing a consensus genomic sequence of PRRSV-2. We demonstrated that our synthetic PRRSV strain confers unprecedented levels of heterologous protection. However, the synthetic PRRSV strain at passage 1 (hereafter designated CON-P1) is highly virulent and therefore, is not suitable to be used as a vaccine in pigs. In the present study, we attenuated CON-P1 by continuously passaging the virus in MARC-145 cells, a non-natural host cell line. Using a young pig model, we demonstrated that the synthetic virus at passages 90 and 122 (designated as CON-P90 and CON-P122, respectively) were fully attenuated, as evidenced by the significantly reduced viral loads in serum and tissues and the absence of lung lesion in the infected pigs. Most importantly, CON-P90 confers similar levels of heterologous protection as its parental strain CON-P1. Taken together, the results indicate that CON-P90 is an excellent candidate for the formulation of next generation of PRRSV MLV vaccines with improved levels of heterologous protection.

1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is one of the most important viral pathogens currently affecting swine production worldwide. The virus infects pigs of all ages; however, it causes more severe clinical manifestations when infecting young pigs and pregnant sows (reviewed in [1]). According to the recently updated and amended taxonomy, PRRSV belongs to genus *Porartevirus*, family *Arteriviride*, and order *Nidovirales* [2]. There are two species of PRRSV: PRRSV-1 and PRRSV-2. These two species share approximately 65% sequence identity [3,4]. The genetic variation among PRRSV isolates within each species is substantial. Phylogenetically, PRRSV-2 isolates are classified into nine lineages, with the average genetic distances between two lineages varying from 11 to 18% [5]. PRRSV-1 isolates are classified into four subtypes, with the subtype 1 is further divided into 13 clades [6]. Multiple factors have been hypothesized to be the driving forces for the remarkable diversity of PRRSV [7].

Multiple types of PRRSV vaccines (e.g. modified live virus (MLV) vaccines, killed virus (KV) vaccines and subunit vaccines) are commercially available; of these, MLV vaccines are considered the most effective (reviewed in [8]). All PRRSV MLV vaccines currently licensed for clinical applications are produced by successive passaging of naturally occurring PRRSV strains on non-natural host cell lines such as MARC-145 cells (Review in [9]) or on a recombinant cell line stably expressing porcine CD163, a key receptor for PRRSV infection [10,11]. One major limitation of the current MLV vaccines is that they do not provide optimal levels of heterologous protection against divergent PRRSV isolates circulating in the field, presumably due to the substantial genetic variation of PRRSV (reviewed in [8,12]).

To overcome the genetic variation challenge, we recently generated a fully synthetic PRRSV strain containing a consensus genome sequence deduced from a set of 59 non-redundant full-genome sequences of PRRSV-2. We demonstrated that the synthetic consensus virus at passage 1 (hereafter designated CON-P1), while highly virulent, can provide unprecedented levels of heterologous protection to the convalescent animals [13]. In the present study, we describe the attenuation and evaluation of the protective efficacy of the attenuated PRRSV-CON.

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2. Material and methods

2.1. Cells, viruses and antibodies

MARC-145 cells, a monkey kidney cell line [14], were used for propagation and titrations of PRRSV. The synthetic CON-P1 was described previously [13]. PRRSV strain MN184C was kindly provided by Dr. K.S. Faaberg, from the U.S. National Animal Disease Center.

2.2. Virus attenuation

CON-P1 was attenuated by successively passaging the virus in MARC-145 cells. Briefly, CON-P1 virus stock was diluted in Dulbecco's Modified Eagle's Medium (DMEM) to the ratio of 1:1000 and inoculated to a monolayer of MARC-145 cells plated in a T-25 flask 48 h earlier. After 1 h of adsorption, the virus inoculum was removed and the cell monolayer was replenished with fresh DMEM containing 2% FBS. The cells were incubated at 37 °C in a humidified atmosphere containing 5% CO2. Once early cytopathic effect (CPE) was visible (approximately 48 h post-infection), culture supernatant containing virus was harvested and clarified by centrifugation at 2000*g* for 10 min. The culture supernatant (designated CON-P2) was divided to 0.5 mL aliquots and stored at −80 °C. This procedure was repeated for a total of 122 times.

2.3. Multiple-step growth curve and plaque assay

Multiple-step growth curve and plaque assay were performed in MARC-145 cells as previously described [15,16].

2.4. Genome sequencing

Complete genomic sequences of CON-P90 and CON-P122 were determined by Next Generation Sequencing using Illumina sequencing technologies as described previously [17].

2.5. Pig experiments

Pig experiments conducted in this study were approved by the University of Nebraska-Lincoln (UNL) Institutional Animal Care and Use Committee protocol number 930. All pigs used in these experiments were PRRSV-seronegative and were accommodated in biosecurity level 2 (BSL-2) animal research facilities. The first experiment was to evaluate the levels of attenuation of CON-P90 and CON-P122 respectively. For this experiment, a total of 24 three-week old pigs were randomly assigned into four groups of six pigs. After one week of acclimation, pigs in group 1 were inoculated intramuscularly with 2 mL DMEM medium to serve as a normal control. Pigs in the remaining groups were separately inoculated intramuscularly with $10^{5.0}$ $TCID_{50}$ of CON-P1, CON-P90 or CON-P122. Blood samples were collected at various time-points post-infection (p.i.), and serum samples were extracted and stored at −80 °C for evaluation of viremia levels and seroconversion. At day 14 p.i., all pigs were humanely sacrificed and necropsied. Microscopic lung lesions were examined by a board-certified pathologist in a blind manner as described previously [18]. During necropsy, samples of tonsil and inguinal lymph node (LN) were collected for quantification of viral load in tissues.

The second experiment was to evaluate the levels of heterologous protection of CON-P90 when compared to CON-P1. For this experiment, a total of 18 three-week-old pigs were randomly divided into three groups of six pigs. After one week of acclimation, pigs in group 1 were injected intramuscularly with DMEM medium to serve as a normal control. Pigs in group 2 and 3 were vaccinated by intramuscular inoculation with $10^{5.0}TCID_{50}$ of CON-P1 or CON-P90, respectively. Blood samples were collected periodically for isolation of plasma and peripheral blood mononuclear cell (PBMC). At day 56 post vaccination (p.v.), all pigs including control group were challenged intramuscularly with heterologous PRRSV strain MN184C at a dose of $10^{5.0}$ $TCID_{50}$ per pig. At day 14 post-challenge (p.c.) all pigs were humanely sacrificed and necropsied. Samples of tonsil and inguinal LN were collected for quantification of viral loads in tissues.

2.6. Quantification of viral loads

Viral loads in serum and tissues were measured by real-time reverse transcription PCR (RT-PCR). For the first experiment, viral loads were measured by a commercial RT-PCR kit (Tetracore Inc., Rockville, MD). For the second experiment, viral loads were measured by using two different RT-PCR kits: the commercial RT-PCR kit (Tetracore, Rockville, MD) that detects total viral RNA resulting from primary and challenge infection, and a differential RT-PCR kit [13] that selectively detects viral RNA from challenge infection. Viral loads in serum were reported as $\log_{10}$ copies per mL whereas viral loads in tissues were reported as $\log_{10}$ copy per μg of total RNA used in the RT-PCR reaction. For statistical purposes, samples that had no detectable levels of viral RNA were assigned a value of 0.

2.7. Measurements of immune responses

IFN-α Porcine ProcartaPlex Simplex Kit (ThermoFisher Scientific) was used for quantification of the concentrations of IFN-α in plasma samples. The serum-virus neutralization (SVN) assay was performed as previously described [19] using plasma rather than serum. Results were expressed as the $\log_2$ of the reciprocal of the highest dilution that showed a ≥90% reduction in the number of fluorescent foci presenting in the control wells. The frequencies of IFN-γ secreting cells (IFN-γ SCs) in peripheral blood mononuclear cells (PBMCs) were measured by using an IFN-γ Elispot assay as previously described [20]. For this assay, PBMCs were stimulated with either CON-P1 or MN184C at the dose of 0.1 $TCID_{50}$ per cell.

2.8. Statistical analysis

Viremia data were analyzed by two-way analysis of variance (ANOVA) while viral loads in tissues were analyzed by one-way ANOVA. Tukey's multiple comparisons test was used for comparison among treatments. Lung microscopic scores were analyzed by Kruskal-Wallis test, followed by Dunn's multiple comparisons test. All statistical analysis was done in GraphPad Prism 7.0 (GraphPad Software, Inc).

3. Results

3.1. In vitro growth properties of CON-P90 and CON-P122

CON-P1 was successively passaged in MARC-145 cells for a total of 122 passages. We then characterized the *in vitro* growth properties of the virus at passages 90 and 120 (designated CON-P90 and CON-P122, respectively). CON-P90 and CON-P122 replicated more efficiently in MARC-145 cells than CON-P1 (Fig. 1A). There was no difference in growth kinetics between CON-P90 and CON-P122. Furthermore, plaque assay results consistently showed that CON-P90 and CON-P122 produced larger plaques than CON-P1 (Fig. 1B). Together, the data indicate that CON-P90 and CON-P122 were well adapted for replication in MARC-145 cells.

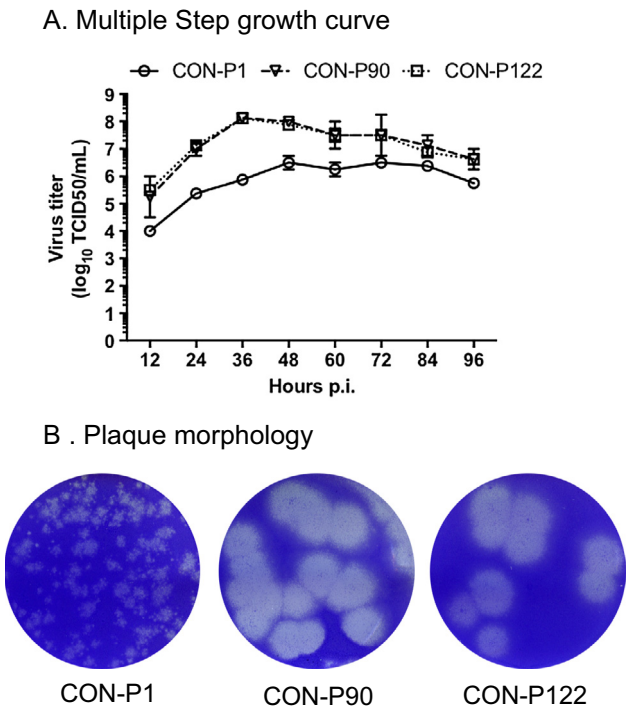

Fig. 1. Growth properties in cell culture. (A) Multiple-step growth curves in MARC-145 cells. Data are expressed as the mean of virus titer and SEM calculated from three independent experiments. (B) Plaque morphology in MARC-145 cells.

3.2. Sequence analysis

The full-length genomic sequences of CON-P90 and CON-P122 were determined. CON-P90 acquired a total of 42 nucleotide mutations (Table 1). Due to the overlapping nature of PRRSV genes, several nucleotide mutations result in amino acid changes in two different proteins. In total, the 42 nucleotide mutations in CON-P90 genome lead to 32 amino acid changes. The nucleotide mutation at position 13,918 results in a premature stop codon in ORF5a protein. Due to this nonsense mutation, ORF5a protein of CON-P90 is four amino acids shorter than that of CON-P1. CON-P122 contained all mutations observed in CON-P90. Furthermore, CON-P122 acquired 13 additional nucleotide mutations, four of which lead to amino acid changes (Table 2). For both CON-P90 and CON-P122, the mutations occurred sporadically throughout the viral genome, except ORF6 and ORF7, where no mutations were found.

3.3. Evaluation of attenuation

The levels of attenuation of CON-P90 and CON-P122 were assessed using a young pig model. As shown in Fig. 2A, pigs in the CON-P90 and CON-P122 groups had significantly lower viremia levels than those in the CON-P1 group. Furthermore, viremia levels of pigs in the CON-P122 group were significantly lower than those in the CON-P90 group, especially at early time-points after infection. All pigs were sero-negative before infection. At day 14 p.i., pigs in the DMEM-group remained sero-negative whereas all pigs in the treatment groups (e.g. CON-P1, CON-P90 and CON-P122) seroconverted (Fig. 2B). Viral loads in inguinal LN and tonsil were not significantly different between CON-P1 and CON-P90 groups, whereas viral loads in tissues were significantly lower in CON-P122 group (Fig. 2C). In regard to lung pathology, pigs in the CON-P90 or CON-P122 groups had significantly less severe lung lesions than those in the CON-P1 group (Fig. 2D). There was no significant difference in lung pathology scores between the CON-P90 and CON-P122 groups. Collectively, the results of this experiment indicate that both CON-P90 and CON-P122 are fully attenuated.

Table 1
Mutations found in both CON-P90 and CON-P122 genomes.

Nucleotide number[a]	Nucleotide change	Protein affected	Amino acid number[b]	Amino acid change
386	C → T	nsp1α	65	Silent
513	G → A	nsp1α	108	A → T
848	G → T	nsp1α	129	K → N
937	T → C	nsp1β	249	V → A
944	G → A	nsp1β	251	M → I
2098	C → T	nsp2	636	A → V
2110	A → G	nsp2	640	D → G
2198	T → C	nsp2	669	Silent
2209	T → C	nsp2	673	V → A
2633	C → T	nsp2	814	Silent
2776	A → T	nsp2	862	E → V
2780	T → G	nsp2	863	D → E
2845	C → T	nsp2	885	P → L
3146	T → C	nsp2	985	Silent
3460	A → G	nsp2	1090	D → G
3474	T → C	nsp2	1095	F → L
3527	G → T	nsp2	1112	Silent
3742	C → T	nsp2	1184	T → I
3849	A → G	nsp2	1220	K → E
3986[d]	A → T	nsp2	1265	Silent
		nsp2TF	1266	Y → F
4389[d]	T → C	nsp2	1400	S → P
		Nsp2TF	1400	Silent
5409	T → C	nsp3	1740	Silent
5984	G → A	nsp4	1931	Silent
6815	A → G	nsp7α	2208	Silent
6902	C → T	nsp7α	2237	Silent
7793	A → T	nsp9	2535	T → S
8326	G → A	nsp9	2712	Silent
8951	G → T	nsp9	2921	A → S
9970	C → T	nsp10	3260	Silent
10334	C → T	nsp10	3382	L → F
10648	A → G	nsp10	3486	Silent
10781	A → T	nsp10	3531	T → S
10799	G → A	nsp10	3537	V → M
12045	T → C	nsp12	3952	V → A
12104[d]	G → T	GP2	10	L → F
		E	9	D → Y
12133[d]	T → A	GP2	20	L → H
		E	18	Silent
12249[d]	T → C	GP2	59	Y → H
		E	57	V → A
12377	T → C	GP2	101	Silent
13199	T → C	gp3	168	Y → H
13373[d]	T → C	GP3	226	Silent
		GP4	44	F → S
13814[d]	G → A	GP5	9	G → S
		ORF5a	12	G → E
13918[d]	C → T	GP5	43	Silent
		ORF5a	47	Nonsense[c]

[a] Nucleotide numbers are based on PRRSV-CON genome (Genbank accession number KT894735).
[b] For non-structural proteins (nsp1α – nsp12), amino acid numbers refer to the polyprotein pp1ab sequence of PRRSV-CON. For the nsp2TF, amino acid numbers refer to the sequence of the polyprotein pp1aTF which is expressed via a −2 ribosomal frameshift [31].
[c] This mutation results in a premature stop codon at amino acid number 47, four amino acids upstream of the original stop codon.
[d] These nucleotide mutations result in amino acid changes in two different proteins, due to the overlapping nature of the ORFs.

3.4. Evaluation of heterologous protection

Next, we compared the protective efficacy between CON-P90 and CON-P1, using a challenge model previously used in our labo-

Table 2
Mutations found only in CON-P122.

Nucleotide number[a]	Nucleotide change	Protein affected	Amino acid number[b]	Amino acid change
139	A → G	5′UTR	N/A	N/A
2362	C → T	nsp2	724	A → V
3279	G → A	nsp2	1030	G → R
5169	A → G	nsp3	1660	I → V
9300	A → G	nsp9	3037	Q → R
9475	C → T	nsp9	3090	Silent
10,525	C → T	nsp10	3445	Silent
10,645	T → A	nsp10	3485	Silent
10,975	C → T	nsp11	3595	Silent
11,029	A → G	nsp11	3613	Silent
12,332	T → C	GP2	86	Silent
12,629	T → C	GP2	185	Silent
13,761	C → T	GP4	173	Silent

[a] Nucleotide numbers are based on PRRSV-CON genome (Genbank accession number KT894735).
[b] For non-structural proteins (nsp1α – nsp12), amino acid numbers refer to the polyprotein pp1ab sequence of PRRSV-CON. For the nsp2TF, amino acid numbers refer to the sequence of the polyprotein pp1aTF which is expressed via a −2 ribosomal frameshift [31].

ratory [13]. All pigs in the DMEM group displayed high viremia levels starting from day 1 p.c., regardless of which RT-PCR kits were used for quantification of viral RNA (Fig. 3A). In contrast, only a portion of pigs in the CON-P1 and CON-P90 groups carried low levels of plasma viral RNA after challenge infection. Specifically, when the commercial RT-PCR kit was used for quantification of viremia, two pigs in the CON-P1 group and three pigs in the CON-P90 group displayed low levels of viral RNA at day 0 p.c., and these pigs continued to show low levels of viremia after challenge infection (Fig. 3A). When the differential RT-PCR kit was used, no pigs in the CON-P90 group had a detectable level of plasma viral RNA while only one pig in the CON-P1 group tested positive by this RT-PCR kit at days 1, 4, 7 and 10 p.c. (Fig. 3A). Overall, the levels of viremia after challenge infection were not statistically significant difference between the CON-P1 and CON-P90 groups, regardless of which RT-PCR kits were used.

In regard to viral load in tissues, the commercial RT-PCR kit detected viral RNA in inguinal LN and tonsil in all pigs but the levels of viral RNA in the CON-P1 and CON-P90 groups were significantly lower than those in the DMEM group (Fig. 3B). When the differential RT-PCR kit was used, only one pig in the CON-P1 group and the CON-P90 group had detectable level of MN184C-specific RNA in tissues whereas all pigs in the DMEM group exhibited high levels of viral RNA (Fig. 3C). Collectively, the results demonstrated that the attenuated CON-P90 maintains the broadly protective phenotype of its parental strain CON-P1.

3.5. Immune responses

We previously reported that CON-P1 induces type I IFNs in cultured cells [15]. In the present study, we sought to determine if this synthetic PRRSV strain induced type I IFN response in pigs. As shown in Fig. 4A, pigs in the DMEM group had very low levels of plasma IFN-α, whereas those in the CON-P1 and CON-P90 groups had high levels (in the range of 40–80 pg per mL) of IFN-α at days 2 and 4 p.v. CON-P90 group appeared to have higher levels of IFN-α than CON-P1 group; however, the difference in IFN- α levels between these two groups was not statistically significant (Fig. 4A).

Subsequently, we measured neutralizing antibodies (NAbs) titers against CON-P1 and MN184C. NAbs against CON-P1were detected in both CON-P1 and CON-P90 groups starting at day 28 p.v. and the titers continued to increase thereafter. At the day of challenge infection (e.g. day 56 p.v.), the geometric mean titers in CON-P1 and CON-P90 groups were 2.58 $\log_2$ and 3.32 $\log_2$, respectively. No significant difference between these two groups was observed in regard to the NAb titers measured against CON-P1 virus (Fig. 4B, left panel). Pigs vaccinated with CON-P1 and CON-P90 mounted meager NAb titers against MN184C. At the day of challenge infection, three pigs in CON-P90 group displayed a titer of 2 $\log_2$ against MN184C whereas no pigs in CON-P1 group had NAb against this PRRSV strain (Fig. 4B, right panel).

Finally, we evaluated the frequencies of IFN-γ SC in PBMCs, using CON-P1 and MN184C as the stimulating antigens. Pigs in CON-P1 group had high frequencies of IFN-γ SC specific to both CON-P1 and MN184C at days 28 and 42 p.i, after which the frequencies of IFN-γ SC decreased (Fig. 4C). Pigs in CON-P90 group had significantly lower numbers of IFN-γ SC than those in CON-P1 group. Interestingly, pigs in CON-P90 group have significantly lower numbers of IFN-γ SC specific to CON-P1 antigen than those specific to MN184C antigen. The was only minimal anamnestic T-cell responses after challenge infection.

4. Discussion

Two different approaches have been used to develop MLV vaccines against PRRSV: serial passaging of a virulent PRRSV strain in a non-natural host cell line (Reviewed in [9]), and molecularly modifying the genome of a virulent PRRSV strain through the use of reverse genetics [21,22]. Currently, all MLVs licensed for clinical applications are generated following the former approach (reviewed in [9]). Typically, it requires approximately 85–100 passages in MARC-145 cells for fully attenuation of virulent PRRSV strains [23–25] while further passaging might result in over attenuation of the vaccine strains, leading to the reduction of vaccine immunogenicity [26]. In the current study, we passaged our synthetic PRRSV strain in MARC-145 cells for a total of 122 passages. We observed that both CON-P90 and CON-P122 are fully attenuated. CON-P90 and CON-P122 acquired 32 and 36 amino acid mutations, respectively. The numbers of mutations in CON-P90 and CON-P122 genomes are in the range observed in naturally occurring PRRSV strains when they are attenuated by passaging in MARC-145 cells [25,27]. The mutations occur sporadically throughout the genome of CON-P90 and CON-P122. It is not trivial to precisely attribute which mutations are associated with the attenuation phenotype of our synthetic PRRSV strain. We believe that a combination of multiple mutations in various genes are responsible for the attenuation of this synthetic virus because our previous studies on naturally occurring PRRSV strains demonstrated that genetic determinants of PRRSV virulence reside in both structural and non-structural genes [28].

Both CON-P90 and CON-P122 can be used for the development of a MLV vaccine as they are both fully attenuated. In this study, we chose CON-P90 as a candidate for evaluation of heterologous protection although CON-P122 might also be an excellent candidate. Under our experimental conditions, pigs vaccinated with MLV vaccines followed by challenge infection with virulent PRRSV strains do not display significant clinical signs. Thus, we primarily use virological parameters for evaluation of protection. We observed in our previous study that pigs recovered from an infection with CON-P1 (virulent) were fully protected from a subsequent infection with heterologous PRRSV strains, demonstrated by the absence of RNA of the challenge PRRSV strains in tissue samples of the pigs [13]. Consistent with our previous observation, in the current study, RNA specific to MN184C, the viral strain used for challenge infection, was not detected from five of six pigs in the

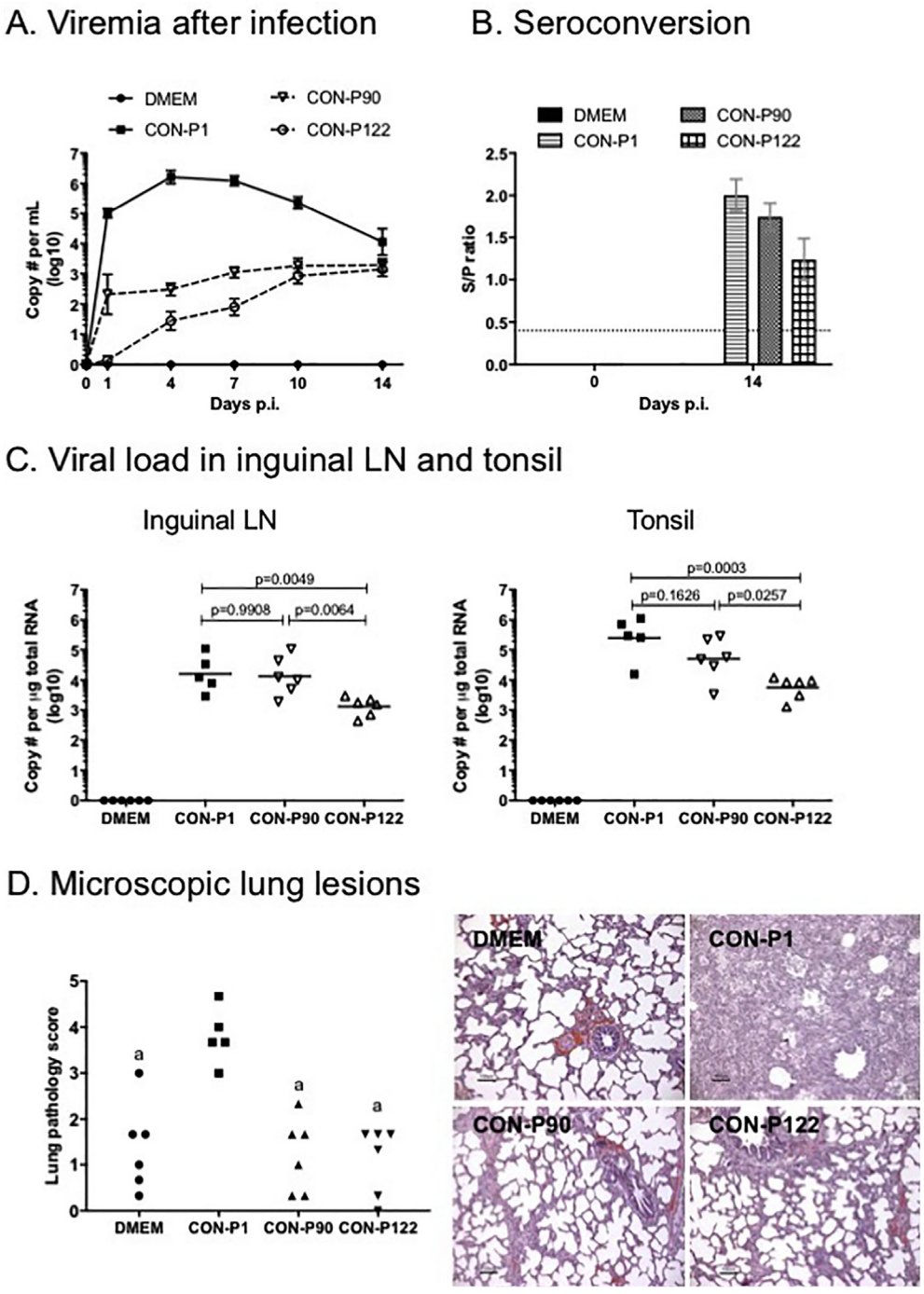

Fig. 2. Evaluation of the levels of attenuation in pigs. A total of 24 PRRSV-negative pigs, three weeks of age, were randomly assigned to four treatment groups. After one week of acclimation, pigs were intramuscularly injected with cell culture medium (DMEM: control group) or with different PRRSV strains at the dose of $10^{5.0}$ $TCID_{50}$ per pig. (A) Viremia after infection, determined by a commercial RT-PCR kit (Tetracore Inc., Rockville, MD). (B) Antibody response measured by IDEXX PRRS X3 Antibody Test (IDEXX Laboratories, Inc., Westbrook, ME). The horizontal dotted line indicates the cutoff of the assay. (C) Viral RNA levels in inguinal lymph node (LN) and tonsil collected at day 14 p.i., determined by a commercial RT-PCR kit. (D) Microscopic lung scores evaluated at day 14 p.i. Left panel shows the lesion score, using a scale from 0 to 5, where 0 is normal and 5 is the most severe lesion. Treatments with the same superscripts are not statistically different ($P > .05$). Right panel shows the representative lung microscopic lesion of pigs each treatment group. Data presented in panels A, B &D are mean and SEM calculated from six pigs in each treatment group.

CON-P1 group (Fig. 3). Most importantly, MN184C-specific RNA was only detected from inguinal LN of one pig (out of six) in the CON-P90 group (Fig. 3C). Thus, the results demonstrated that the attenuated CON-P90 confers equivalent levels of heterologous protection against MN184C as compared to the virulent strain CON-P1.

Both NAbs and IFN-γ SC are believed to be components of the protective immunity against PRRSV [29]. In the present study, pigs vaccinated with CON-P1 and CON-P90 did not mount significant NAbs against MN184C, the challenge virus. In contrast, relatively high frequencies of MN184C-specific IFN-γ SC (average 50 cells per 1 million PBMCs) were observed in both CON-P1 and CON-P90 groups at the day of challenge infection (day 56 p.v.). Thus, in the context of this study, IFN-γ SC is more likely a correlate of the immune protection against PRRSV infection. One interesting

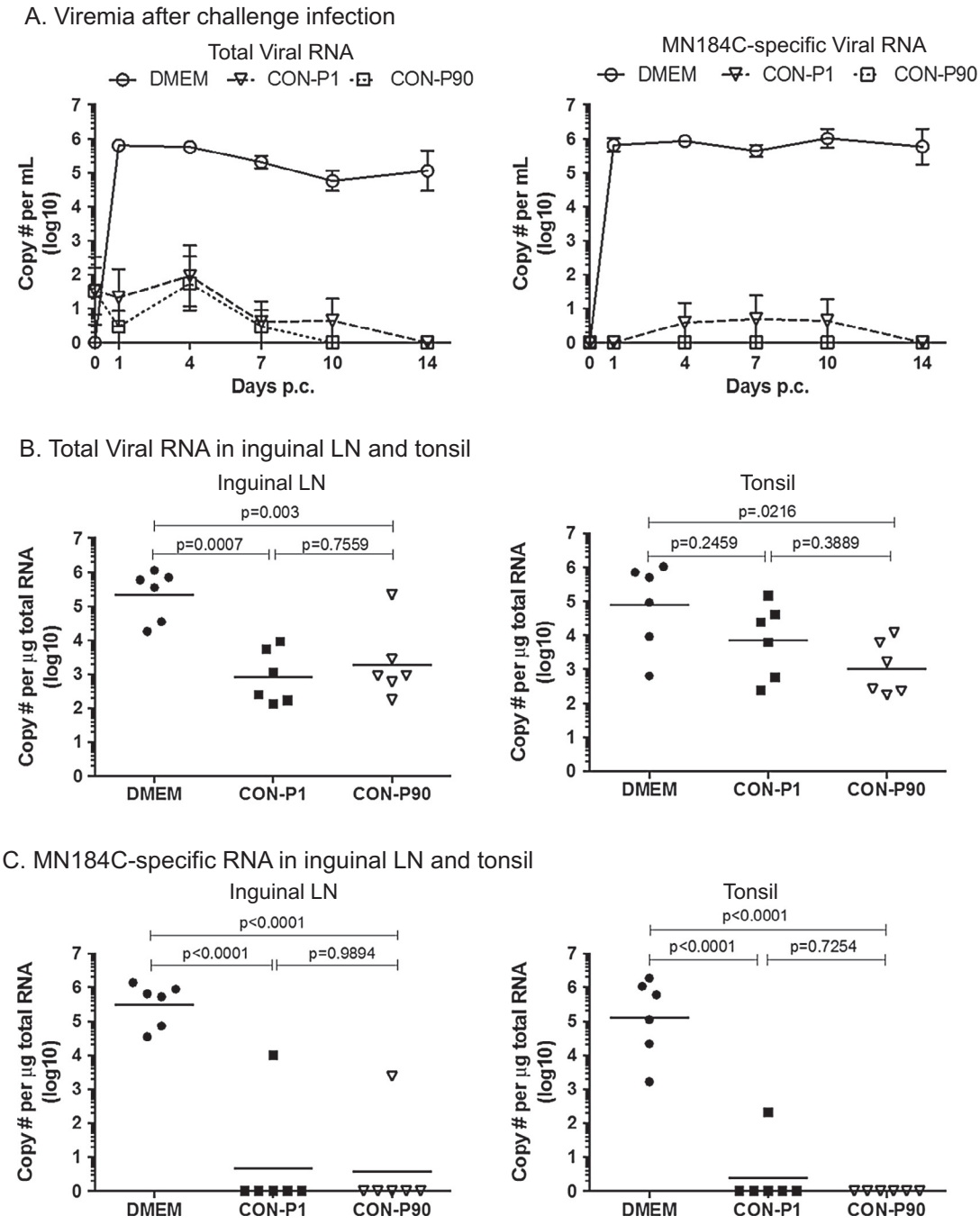

Fig. 3. Evaluation of heterologous protection. A total of 18 three-week-old PRRSV-seronegative pigs were assigned into three treatment groups. After one week of acclimation, pigs were intramuscularly injected with cell culture medium (DMEM: control group) or with CON-P1 or CON-P90 at the dose of $10^{5.0}$ $TCID_{50}$ per pig. At day 56 post-vaccination (p.v.), the pigs were challenged by intramuscular inoculation with $10^{5.0}$ $TCID_{50}$ of a heterologous PRRSV strain MN184C. (A) Viremia post-challenge infection (p.c.) measured by two different RT-PCR kits: a commercial RT-PCR kit (Tetracore Inc., Rockville, MD) detecting virtually all viral RNA and a differential RT-PCR kit detecting only MN184C-specific RNA. Data are expressed as mean and SEM calculated from six pigs in each treatment group. (B) Viral RNA in inguinal lymph node (LN) and tonsil at day 14 p.c. (day 70 p.v.), quantified by a commercial RT-PCR kit. (C) Viral RNA in inguinal lymph node (LN) and tonsil at day 14 p.c. (day 70 p.v.), quantified by a differential RT-PCR kit.

observation is that the pigs in CON-P90 group had greater numbers of IFN-γ SC specific to MN184C than numbers of the IFN-γ SC specific to CON-P1. At the present, we do not have any explanations for this observation.

PRRSV can infect pigs of all ages; however, the virus causes more prominent clinical manifestations when infecting young pigs and pregnant sows. We demonstrated in this study that CON-P90 is attenuated and capable of conferring heterologous protection using a young pig model. In the future, we will assess the attenuation and protective efficacy of CON-P90 in pregnant sows. Additionally, we will need to evaluate the stability of CON-P90 after multiple passages in pigs to ascertain that the virus does not revert back to virulence.

In summary, the synthetic PRRSV-CON is successfully attenuated after 90 passages in MARC-145 cells and that the CON-P90 maintains the protective phenotypes of the

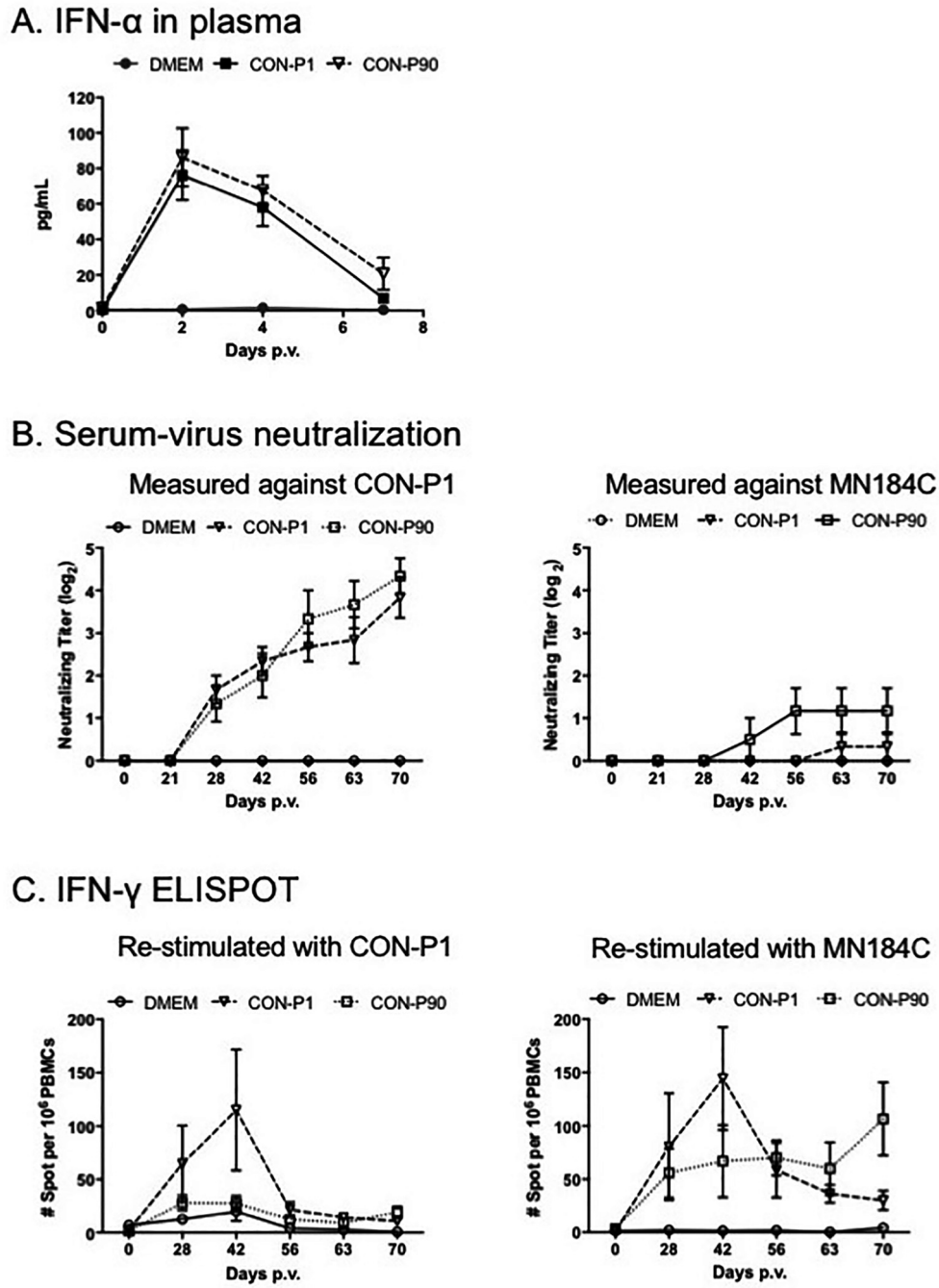

Fig. 4. Immune responses. (A) Concentration of IFN-α in plasma during the first week post-vaccination (p.v.). (B) Serum-virus neutralizing antibodies measured against CON-P1 and MN184C. (C) Frequencies of IFN-γ secreting cells in PBMCs measured by IFN-γ ELISPOT with CON-P1 and MN184C as the simulating antigens. Date presented in this figures are mean and SEM calculated from six pigs in each treatment group.

parental strain. This study demonstrates the feasibility of the application of synthetic virology [30] to vaccine research and development.

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